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16. ABSTRACT

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Changes in Accessibility of DNA to Various Fluorochromes During Spermatogenesis

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Accessibility of mouse testicular and vas deferens (vas) sperm cell DNA to acridine orange, propidium iodide, ellipticine, Hoechst 33342, mithramycin, chromomycin A_3, 4'6-diamidino-2-phenylindole (DAPI), and 7-amino-actinomycin D (7-amino-AMD) was determined by flow cytometry. Permealized cells were either stained directly or after pretreatment with 0.06 N HCl. For histone-containing tetraploid, diploid, and round spermatid cells, HCl extraction of nuclear proteins caused an approximately sixfold increase of 7-amino-AMD stainability but had no significant effect on DAPI stainability. For these same cell types, the stainability with other intercalating (acridine orange, propidium iodide, ellipticine) and externally binding (Hoechst 33342, mithramycin, chromomycin A3) dyes was increased by 1.6- to 4.0-fold after HCl treatment. In sharp contrast, HCl treatment of vas sperm did not increase the staining level of 7-amino-AMD, DAPI, or propidium iodide but did increase the staining level for the other intercalating dyes (1.3- to 1.5-fold) and external dyes (1.3- to 1.9-fold). Elongated spermatids that contain a mixture of protein types including histones, transition proteins, and protamines demonstrated the greatest variability of staining with respect to type of stain and effect of acid extraction of proteins. In general, for nearly all dyes, the round spermatids had an increased level and tetraploid cells had a decreased level of stainability relative to the same unit DNA content of diploid cells. The observed differential staining is discussed in the context of chromatin alterations related to the unique events of meiosis and protein displacement and replacement during sperm differentiation.

Key terms: Flow cytometry, spermatogenesis, chromatin, nuclear proteins, differential DNA dye binding

The mammalian spermatogenic cycle begins with stem cell or A-type spermatogonia that divide to produce primary spermatocytes. Meiotic division of these spermatocytes results in the production of two daughter cells, secondary spermatocytes, which each divide to give rise to four spermatids. Spermatozoa represent the final step of a complex pathway of cellular differentiation.

Chromatin alterations occur as cells undergo mitosis and the unique event of meiosis. Of particular interest here, spermatid chromatin undergoes dramatic alterations including replacement of nuclear histones by arginine-rich sperm specific nuclear protamines (25,30), condensation of nuclear chromatin, and the formation of S-S crosslinks between nuclear proteins (5). During spermiogenesis, a progressive restriction in accessibility of DNA to various ligands occurs. For instance, binding of the intercalating molecules acridine orange (AO) (11,15,16) and actinomycin D (7) to DNA is decreased during maturation of sperm cells. A discussion of factors, including differentiation, involved in restricting accessibility of DNA in somatic cells was presented in a recent publication (8). Alteration of chromatin structure is more complex for spermatogenesis where extensive biochemical changes occur in chromatin including reorganization from a nucleosome structure to a smooth fiber (1,21). Flow cytometric (FCM) analysis of abnormal sperm chromatin structure has been reported (10,12,13).

The dramatic loss of DNA stainability for differentiating testicular cells has been observed previously by microspectrophotometric (15) and FCM measurements of testicular cells. Previous FCM measurements of testicular cells have utilized AO (14), propidium iodide (PI)(26), ethidium bromide (EB)(6,29), a combination of EB-mithramycin (18), or the acriflavin-Feulgen method (27). The

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latter study initially demonstrated that various testis nuclei, identified by FCM sorting, did not undergo stoichiometric staining relative to the nuclear DNA content. In these other studies, sample preparations were different than used here, e.g., cell fixation and pepsin-HCl digestion. Comparison between eight DNA dyes commonly used in flow cytometry (22) can more easily be made in this study since the samples, prepared in two ways, essentially differed only by the dye used for staining. Furthermore, we analyzed the effect of HCl-extractable chromatin constituents on DNA accessibility to the probes at various stages of sperm differentiation. The modes of interaction of these fluorochromes with DNA are varied and include external binding, intercalation, and other interactions. Thus, as discussed before (8), PI and ellipticine are strong, "simple" intercalators, differing, however, in the degree of DNA unwinding. Acridine orange, in addition to intercalation, forms complexes with single stranded nucleic acids characterized by metachromatic luminescence. 7-amino-actinomycin D (7-amino-AMD) has the intercalating chromophore and a large peptide moiety that upon binding to DNA interacts with the minor groove of the double helix. Hoechst 33342, mithramycin, and chromomycin A_3 are externally binding dyes. There is a controversy regarding the mechanism of DAPI binding; most likely it intercalates but produces minimal unwinding. Interactions of these different dyes with chromatin, therefore, may provide information about changes in chromatin structure during differentiation. Furthermore, because most of these dyes are commonly used in cytofluorometry, the knowledge of DNA stainability (restriction of binding) in relation to different stages of spermatogenesis may be helpful in identification of particular populations of testicular cells.

MATERIALS AND METHODS

Animals

Six-week-old F_1 male mice (C57BL/6J female × C3H/HeJ male) were obtained from the Jackson Laboratory (Bar Harbor, ME) and kept for 10 wk prior to sacrifice. The mice were housed in plastic cages with wire mesh tops and allowed free access to Purina Certified Rodent Laboratory Chow (#5002) and deionized water. Room temperature was maintained at 21 ± 2°C; lighting was on a 0700–1900-hr schedule. The mice had an average body weight of 30 g. Animals were killed by cervical dislocation.

Cells

Immediately following cervical dislocation, the testes, epididymides, and vas deferentia (vas) were excised and placed into Hank's balanced salt solution (HBSS;4°C). The testes were minced with curved scissors for approximately 1 min until only small fragments remained; then the sample was transferred to a 12-×-75-mm conical tube. After letting the small tissue fragments settle out for several minutes, the cellular suspension was transferred to a 1-ml tuberculin syringe with 53-μm nylon mesh (Tetko, Inc., New York, NY) between the lower end of the syringe and the protective cap (tip removed with a wire cutter). The filtrate was diluted as necessary with HBSS to produce a cell concentration of approximately 2×10^6 cells/ml. This procedure provides a representative distribution of testicular cell types that was not significantly altered when the minced testicular population was incubated with trypsin, collagenase, and deoxyribonuclease I (data not shown).

Sperm were liberated from the vas by pressing a blunt dissecting probe along the length of the tubule submerged in HBSS. The "cords" of sperm were picked up with a Pasteur pipette and the suspended cells were filtered as above through a 153-μm nylon mesh. These testicular and vas sperm samples were kept on ice and used for up to 4 hr after preparation with no significant alterations seen in cell stainability.

Cell Staining

Testicular cells were treated with RNAse prior to staining by admixing equal volumes of cell suspension and HBSS containing 0.1% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO) at 4°C. RNase A (RNase, Worthington Biochem. Co., Freehold, NJ) was added to the cell suspension to a final concentration of 10^3 units/ml; the mixture was incubated at 23°C for 30 min and then returned to 4°C.

Testicular cell suspensions with or without added vas sperm were stained with each dye either directly (one-step method) or after pretreatment with HCl (two-step method) as previously described (9,11). In the one-step method, 0.20-ml aliquots of cells were admixed with 2.0 ml of the respective dye solution in 0.1 M PIPES buffer (pH 6.4), 2 mM $MgCl_2$, and 0.1% Triton X-100 at 4°C. For the two-step method, 0.20-ml aliquots of cells were admixed with 0.40 ml of a solution containing 0.08 N HCl, 0.15 M NaCl, and 0.1% Triton X-100 for 30 sec at 4°C (the final HCl concentration was 0.06 N, pH 1.6) and then admixed with 2.0 ml of a dye solution in 0.1 M PIPES buffer (pH 6.4), 2 mM $MgCl_2$, and 0.1% Triton X-100 at 4°C. The staining reaction was very rapid for all dyes used except mithramycin, chromomycin A_3, and Hoechst 33342, which required up to 30 min for equilibration. Dye concentrations used were those previously used for somatic cells (8) with the dye/phosphate ratio ≤2.0 at staining equilibrium. The fluorochromes and concentrations used were as follows: AO (chromatographically purified), 5 μg/ml; Hoechst 33342, 0.12 μg/ml; PI, 10 μg/ml; 4'6-diamidino-2-phenylindole (DAPI), 2 μg/ml (all obtained from Polysciences, Warrington, PA); 7-amino-AMD, 10 μg/ml, and chromomycin A_3, 10 μg/ml (both obtained from Calbiochem-Behring Co., La Jolla, CA; mithramycin, 10 μg/ml (Sigma Chemical Co., St. Louis, MO) and ellipticine (5,11-dimethyl-6 H-pyrido 4,3-b-carbazole), 5 μg/ml, provided by Dr. David Abraham, Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. We have made the assumption that the dye staining was specifically re-

stricted to DNA and the cells were fully permeable to the dyes, and thus the measurements were a reflection of chromatin stainability at equilibrium (8). It was also assumed (8) that the HCl treatment removes histone H1 and in all probability most of the nucleosome core histones (review, 19).

Flow Cytometry

Stained cells were individually measured in an ICP22A flow cytometer interfaced to an Ortho Diagnostics 2150 data handling system (Ortho Diagnostics, Inc., Westwood, MA) and a Hewlett Packard 7015 X, Y plotter (Hewlett Packard Co., San Diego, CA). Measurement of the fluorescence of cells stained with DAPI and Hoechst 33342 utilized the UGl excitation filter and a combination of dichroic mirrors and secondary filters transmitting light between 450 and 490 nm (FITC blue filter). Cells stained with mithramycin, chromomycin A_3, AO, and ellipticine were excited with the BG12 filter. For mithramycin and chromomycin A_3, light transmitted through a 550-nm (orange) long-pass filter was measured. For AO and ellipticine, measurements were made of light transmitted through a 515–530-nm band-pass filter (FITC green).

Cells stained with 7-amino-AMD and PI were measured in a Cytofluorograf II equipped with a 100-mW Lexel argon-ion laser (488 nm) and interfaced to the same 2150 data handling system described above. The AO measurements were made on both the Cytofluorograf II and ICP22A. The data are based on 0.5–1 $\times$ 10^4 cells per sample.

Measurements were made in pairs: acid- and nonacid-treated samples were consecutively stained and measured at a single photomultiplier tube (PMT) setting. Repeat measurements were independently taken two to ten times for each dye; PMT gains often varied between pairs of measurements.

RESULTS

Figure 1.A and B, shows representative frequency histograms comparing one- and two-step staining of testicular and vas sperm cells for each of the eight dyes. All samples were measured 5 min after staining; chromomycin A_3-, mithramycin-, and Hoechst 33342-stained cells were also measured at 30 min since staining equilibrium was slower for these dyes.

To illustrate the repeatability among different animals and independent measurements, the following peak staining values $\pm$ S.D., normalized to 1.0 for diploid cells, were obtained for well-resolved testicular cell types from five individual mice, stained with either AO or PI: AO values were: elongated spermatid (ES) = 0.39 $\pm$ 0.02, round spermatid (RS) = 0.54 $\pm$ 0.03. The PI values were: ES = 0.40 $\pm$0.04, RS = 0.61 $\pm$ 0.02, tetraploid (T) = 1.91 $\pm$ 0.09. All other data are consistent with this level of repeatability. The resolution of the cell populations was good for all dyes with a CV of the haploid and/or round spermatid peak ranging from 2.9 to 4.0%. Cell types were verified either by previously sorting AO-stained testicular cells (Evenson and Staiano-Coico, unpublished) or by adding vas sperm and/or diploid somatic cells (Friend leukemia cells) to these testicular suspensions. The stainability of each cell type, shown in Figure 2 and 3, was related to the unit DNA content of diploid cells ($diploid_{1c}$). In those cases where more than one diploid peak was resolved, the predominant peak, which most likely represents the same cell type, was used.

When the DNA fluorescence signal in the Cytofluorograf II was electronically processed and displayed as peak amplitude of signal vs. area of signal integrated over time, it was observed that a negligible percentage of a lower ploidy population. The linearity of the instrument was within 4% when calculating the singlet to doublet ratio of stained calf thymocyte nuclei (Ortho Diagnostics, Inc.) or 10-μm fluorescent beads.

For all of the dyes studied, with the exception of PI for non-acid-treated cells, and mithramycin for acid-treated cells, the $tetraploid_{1c}$ population had lower DNA stainability than $diploid_{1c}$ cells. The range was 73–95% and 74–99% for non-acid-treated (Fig. 2) and acid-treated cells (Fig. 3), respectively. However, since the tetraploid population was $\leq$10% of the total cells, use of the peak channel was not highly accurate for these determinations.

In contrast to tetraploid cells, round spermatids had a greater amount of DNA stainability than $diploid_{1c}$ cells for all dyes. The range was 108–134% and 102–120% for non-acid-treated (Fig. 2) and acid-treated (Fig. 3) cells, respectively.

For PI, ellipticine, AO, and 7-amino-AMD, one-step staining did not resolve round from elongated spermatids (Figs. 1 and 2) whereas they were resolved by two-step staining (Figs. 1 and 3). Round and elongated spermatids can be resolved without acid pretreatment for the external binding DNA dyes (Fig. 2). Note that acid pretreatment caused a marked increased stainability of the elongated spermatids relative to $diploid_{1c}$ cells (Fig. 3).

Figure 4 shows the ratio of the peak staining values of acid/non-acid-treated cells. Note the range from DAPI-stained cells, showing only a slight increase after acid treatment, to 7-amino-AMD-stained cells, which showed over a sixfold increase in stainability of DNA.

Highlighted staining properties of each dye are described as follows.

Acridine Orange

Only after acid pretreatment were the elongated spermatids easily distinguished from round spermatids. In addition, a shoulder on the frequency histogram (Fig. 1A), apparently representing elongating spermatids, is seen to the left of the round spermatids. Elongating spermatids can be resolved into a distinct population by using two-parameter (DNA, RNA) AO staining (13,14). Non-HCl and HCl-treated sperm cells had 46 and 16% of the stainability of similarly treated $diploid_{1c}$ cells, respectively (Figs. 2,3). Acid dissociation of histones al-

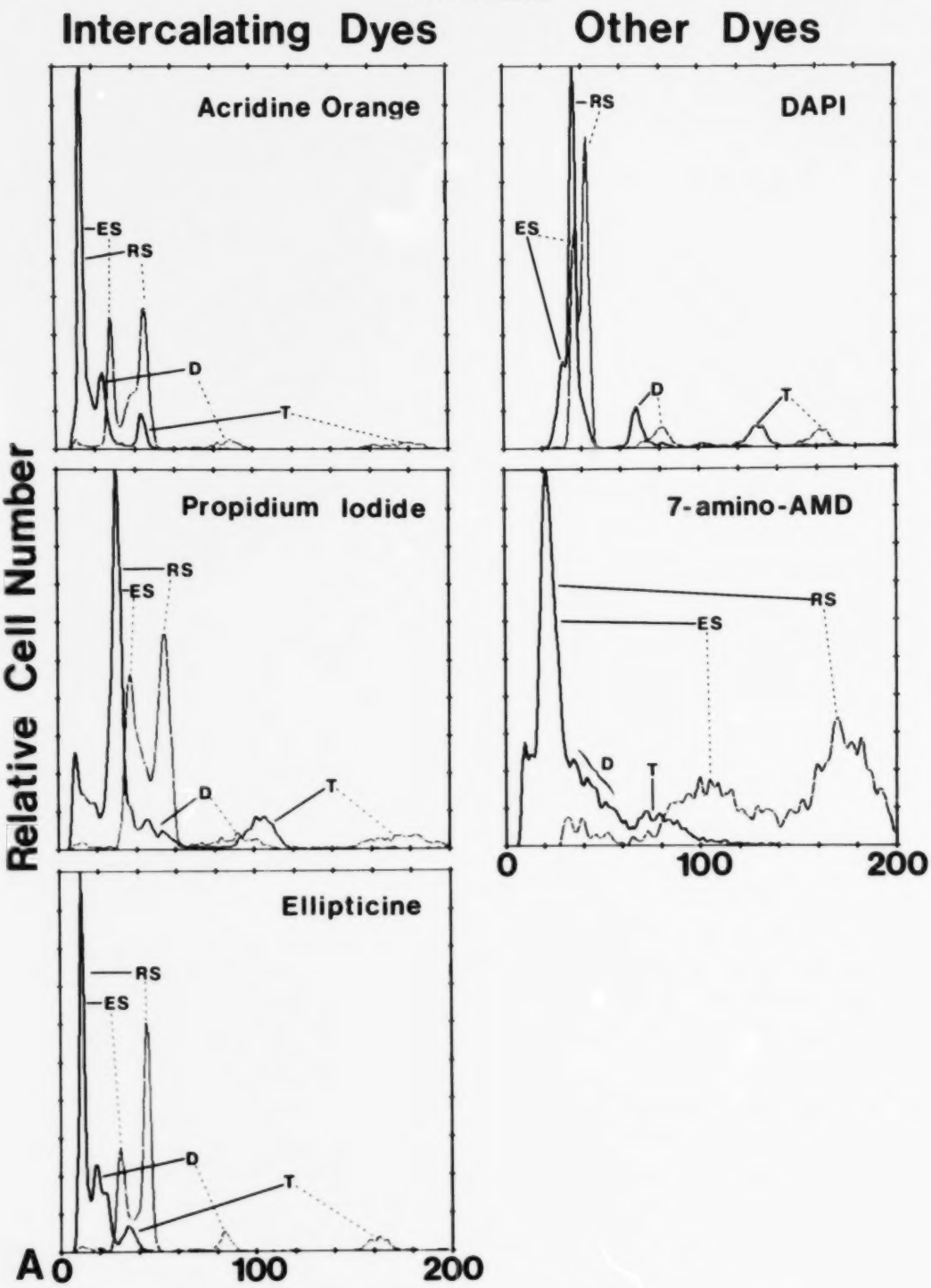
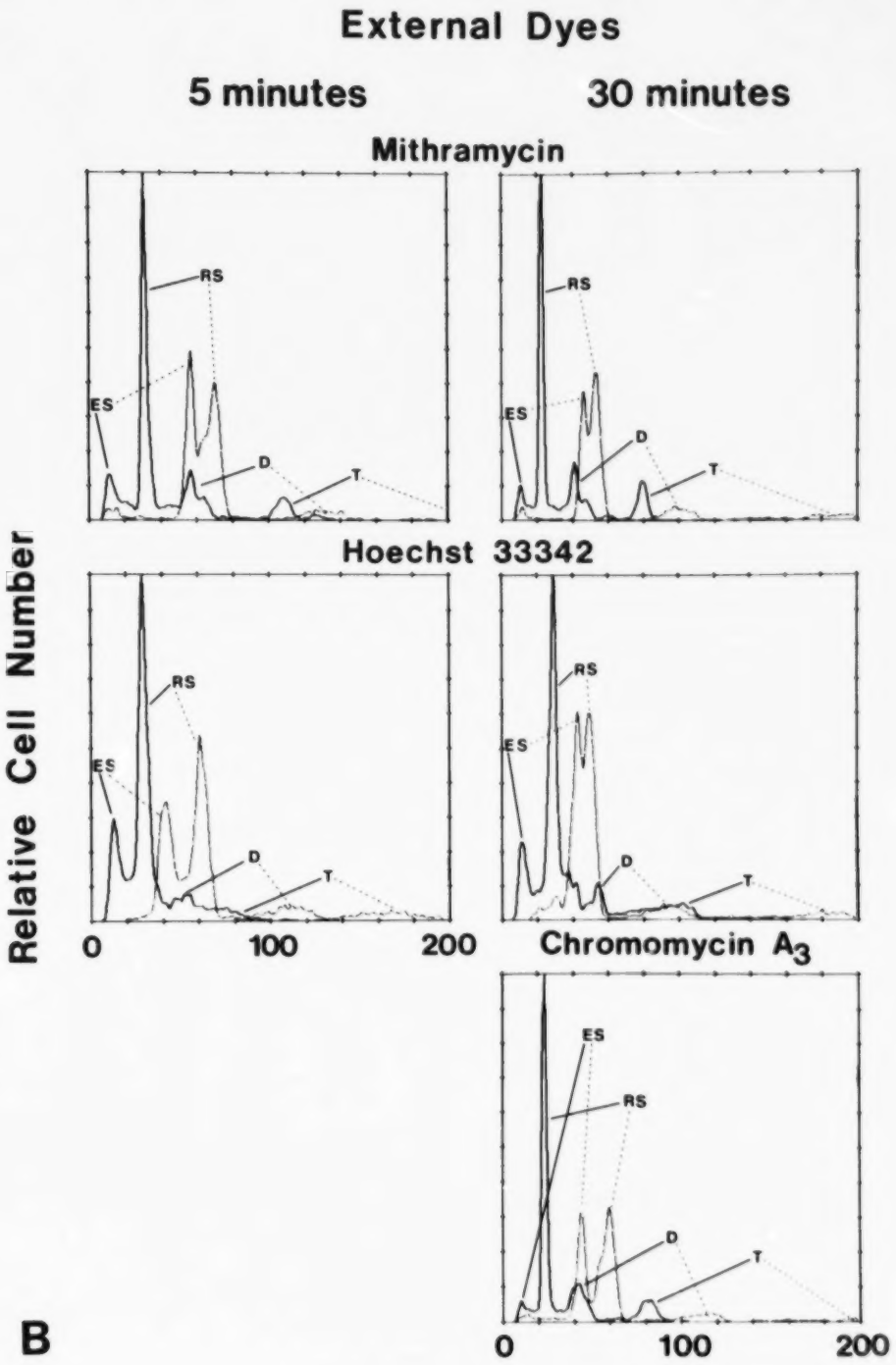


FIG. 1. Frequency histograms (PLOT program courtesy of Dr. Peter Rabinovitch) of testicular cells stained with external or intercalating DNA dyes or dyes that bind to DNA in another manner. Each overlapping frequency histogram compares the stainability of the cells either not treated with HCl (——, one-step method) or treated with HCl (– – –, two-step method). The cell populations (T, tetraploids; D, diploids; RS, round spermatids; ES, elongated spermatids) are pointed out for both HCl- (——) and non- HCl- (—) treated samples.


External Dyes
5 minutes
30 minutes
Mithramycin
Hoechst 33342
Chromomycin A3
Relative Cell Number
ES
RS
D
T
0
100
200
B

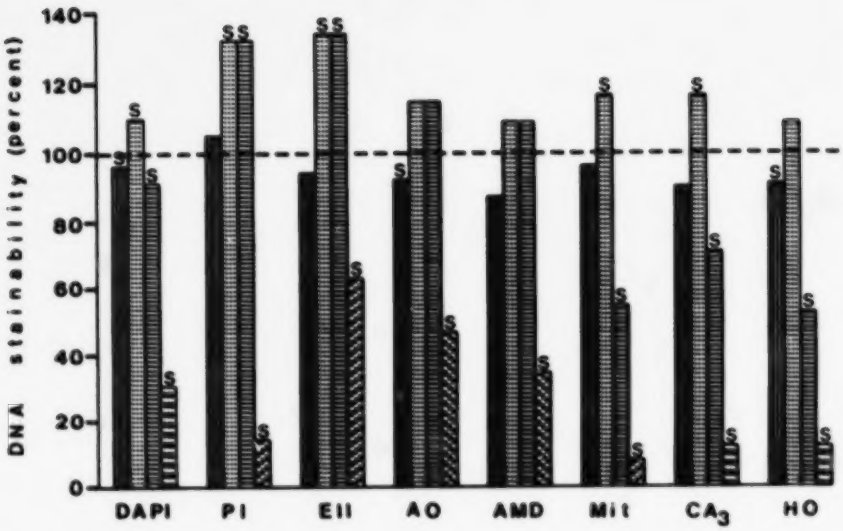


FIG. 2. Relative DNA stainability of fresh, Triton X-permealized, *non-HCl-treated* testicular cells and vas sperm. Peak staining values of all cell types are normalized to unit (1c) content of DNA with diploid cell values set to 100%. Each bar represents the mean peak staining values; "S" at the top of bars indicates statistical significance as defined by being ± 2 standard deviations away from the diploid$_{1c}$ value. Vertical lines, tetraploid cells; dots, round spermatids; horizontal lines, elongated spermatids; dashed vertical lines, vas sperm. Ell, ellipticine; AMD, 7-amino-AMD; Mit, mithramycin; CA_3, chromomycin A_3; HO, Hoechst 33342.

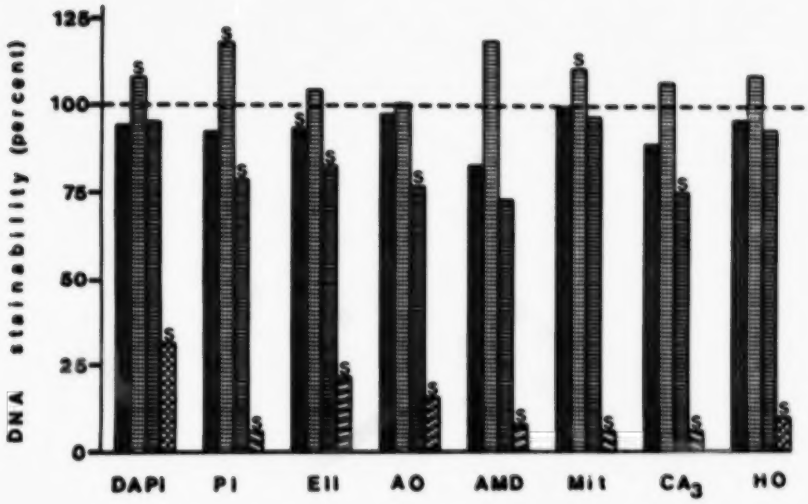


FIG. 3. Relative DNA stainability of fresh, Triton X-permealized, and *HCL-treated* testicular cells and vas sperm. Peak staining values of all cell types are normalized to unit (1c) content of DNA with diploid cell values set to 100%. The bar, dye, and "S" abbreviations represent the same as in Figure 2.

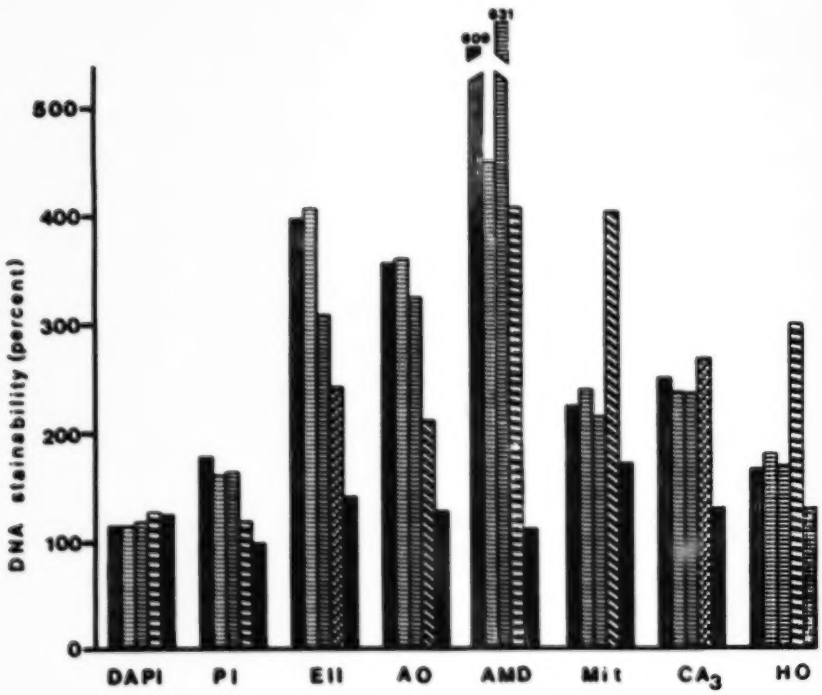


FIG. 4. Percent DNA stainability of fresh, Triton X-permealized, and HCl-treated testicular cells and vas sperm cells relative to the same cell types stained without acid pretreatment. Each value was derived from the mean of all peak fluorescence values obtained for each dye and cell type treated with HCl/not treated with HCl. Vertical lines, diploid cells; dots, tetraploid cells; horizontal bars, round spermatids; dashed vertical lines, elongated spermatids; grid lines, vas sperm. The dye abbreviations are the same as in Figure 2.

lowed for the third highest increase (360%) of DNA stainability of diploid cells of the eight dyes (Fig. 4).

Propidium Iodide

Figure 2 shows a slightly higher stainability of tetraploid$_{1c}$ cells relative to diploid$_{1c}$ cells. Round spermatids had 32% (Fig. 2; non-HCl) and 20% (Fig. 3; HCl) higher stainability than diploid$_{1c}$ cells. Without HCl, round and elongated spermatids were not distinguishable (Fig. 2), whereas with HCl pretreatment, DNA stainability of round and elongated spermatids relative to diploid$_{1c}$ cells was 120 and 80%, respectively (Fig. 3). Vas sperm had 6 and 14% of the stainability of HCl- and non-HCl-treated diploid$_{1c}$ cells, respectively (Figs. 2,3).

Ellipticine

Ellipticine, an intercalating dye, had staining properties similar to those of AO and PI, as seen in Figures 1–4. This dye had the second highest percentage increase of stainability following HCl extraction of basic proteins, with 400 and 410% increases for diploid and tetraploid cells, respectively (Fig. 4). With non-HCl-treated cell populations, round spermatids had the highest amount of stainable DNA relative to diploid$_{1c}$ cells, i.e., 134% (Fig. 2). After HCl, this difference was reduced to 106% (Fig. 3). As with PI and AO, separate peaks for round and elongated spermatids could not be distinguished in the non-acid-treated samples, but were noted after acid treatment. Vas sperm staining intensity after HCl treatment was greater than that of AO or PI relative to diploid$_{1c}$ cell staining intensity (Fig. 3) and also had a greater percentage increase of stainability after acid treatment (Fig. 4).

Hoechst 33342

The staining reaction to equilibrium was slow and measurements were made at 5 and 30 min. For each time point, the data were collected at different PMT settings in order to keep the 30-min values on the same scale as the 5-min values. Of interest, although the ratio of *round* spermatid DNA to diploid$_{1c}$ cell DNA peak value staining stayed approximately the same at the two time points shown in Figure 1B, (5 min—1.10 (non-HCl) vs. 1.20 (HCl); 30 min—1.08 (non-HCl) vs. 1.10 (HCl)),

the ratio of *elongated* spermatids to diploid$_{1c}$ cells was different (5 min—0.48 (non-HCl) vs. 0.84 (HCl); 30 min—0.52 (non-HCl) vs. 0.94 (HCl)). Also, a distinct separation of the round and elongated spermatids (HCl treated) was visible at 5 min, but only a slight separation of the peaks was evident after 30 min (Fig. 1B). Thus, the elongated spermatids pretreated with HCl preferentially took up more dye with time.

Chromomycin A_3/Mithramycin

Mithramycin and its analog chromomycin A_3 are both specific for dG.dC and have similar staining characteristics for tetraploid and diploid cells. However, for elongated spermatids, mithramycin had the highest increase (4.8-fold, 5 min; 4.1-fold, 30 min) of staining after HCl treatment (Figs. 1B,4). Chromomycin A_3 also stained elongated spermatids at a higher level following acid treatment but less than mithramycin and Hoechst 33342. The stainability of vas sperm for all three external dyes was similar.

7-Amino-Actinomycin D

Tetraploid$_{1c}$ cells (non-HCl) exhibited the second lowest (86%; Fig. 2) amount of fluorescence relative to diploid$_{1c}$ cells; round spermatids (non-HCl) had 108% fluorescence of diploid$_{1c}$ cells, one of the smallest values noted. Vas sperm had 34% of the stainability of diploid$_{1c}$ cells (non-HCl). Following HCl extraction, however, round spermatids were still relatively higher than diploids$_{1c}$ (120%) but vas sperm were low (8%). Most dramatic was the very high differential between non-HCl and HCl treatment resulting in a 410, 630, 610, and 450% increase for elongated and round spermatids, diploid and tetraploid cells, respectively (Fig. 4).

DAPI

HCl pretreatment resulted in only a 16% increase of DNA stainability of diploid cells, the lowest increase of the dyes studied; tetraploid cells also showed a 16% increase (Fig. 4). Round spermatids had a 109% (non-HCl) and 110% (HCl) DNA stainability of diploid$_{1c}$ cells (Figs. 2,3). After HCl pretreatment, DAPI had the highest efficiency for staining vas sperm DNA with 32% of diploid$_{1c}$ cells (Fig. 3).

DISCUSSION

Spermatogenesis involves the most dramatic alterations in chromatin structure known to occur in the mammalian system. In this study, a comparative analysis of germ cell staining by eight different DNA dyes was made to determine whether the stainability was differentially affected relative to a) mode of dye binding to DNA, b) alteration of chromatin protein constituents, and c) chromatin condensation.

The binding relationship of nine DNA dyes for undifferentiated and differentiated somatic cells was recently reported (8). For the common dyes, the stainability pattern generally observed for diploid cells in this study was consistent with the previous study. Thus, the emphasis in this paper is on differential DNA stainability of HCl-treated or non-HCl-treated meiotic cells, round and elongating spermatids, and mature sperm relative to alterations of chromatin structure.

Of particular interest in this study is the effect on stainability due to exchange of histones for transition proteins and finally protamines in maturing testicular spermatids and mature vas sperm. Of the four transition proteins found in late-step rat spermatids, at least three are synthesized and deposited on chromatin prior to synthesis and deposition of protamine (17); this is most likely true of mouse also. Mouse sperm contain two sequence variants of protamine (2) whose relative proportions differ between late-step spermatids and mature sperm (3). Transition proteins are intimately associated with DNA or protamine in late-step spermatids (3) but are only transient components, since they are completely removed from the maturing DNA-protamine complex before the sperm moves into the epididymis. These transient proteins are probably reflected in the dramatic differential staining seen between round and elongated spermatids and also between non-HCl- and HCl-treated elongated spermatids (Fig. 4). For external binding dyes, the relative increase in staining after acid treatment was greater for elongated spermatids than for round spermatids, while for intercalating dyes there was a relative decrease and for other dyes no significant relative change (DAPI).

Vas sperm chromatin has a highly condensed structure that restricts DNA binding due in part to the disulfide crosslinks between cysteine groups on protamines (5). These S-S crosslinks most likely inhibit HCl extraction of vas sperm protamines. To some extent this is also true of elongated spermatids in the testes since HCl does not remove all of the protamine and some of the transitional proteins remain behind when HCl extraction is used without disulfide bond reduction (24). Thus, for the intercalating dyes, HCl treatment produced a large relative decrease of sperm to diploid$_{1c}$ cell stainability in most part due to a greater relative *increase* in the stainability of histone-containing diploid$_{1c}$ cells relative to protamine-containing sperm cells (Fig. 3). In contrast, HCl treatment did not change the vas sperm stainability to a significant extent for the external dyes or DAPI. Interestingly, staining with 7-amino-AMD resulted in the greatest relative difference of stainability between non-acid- and acid-treated diploid cells and sperm cells (34%, Fig. 2, vs. 8%, Fig. 3, respectively). This observation may be due to the staining properties of 7-amino-AMD which is known to involve the minor groove of the DNA helix, i.e., the dye binds by intercalation of the chromophore between the G-C bases while the bulky peptide rings locate in the minor groove (20). A recent model (1) for the structure of chromatin in mammalian sperm suggests that protamine is restricted to the minor groove of DNA, thereby neutralizing the phosphodiester backbone and causing a dramatic compaction of the nucleohistone structure. Thus, these and previous (7) data agree with the model (1), suggesting that protamine in the minor groove would greatly restrict 7-amino-AMD binding. A second reason for the differential 7-

amino-AMD stainability of protamine-containing sperm and histone-containing testicular germ cells is the dramatic sixfold increase in stainability of the latter following acid treatment (Fig. 4). In general, for HCl-pretreated cells other than differentiating spermatids and sperm, the external dyes showed an approximate twofold increase in stainability, while the intercalating dyes demonstrated a 1.6–4.6-fold and DAPI about a 1.1-fold increase (Fig. 4).

Round spermatids in all cases exhibited a greater stainability than diploid$_{1c}$ cells, ranging from 2 (AO with HCl) to 34% (ellipticine with HCl; Fig. 4). In general, the intercalating dyes without HCl treatment showed the highest relative ratio. These data suggest that round spermatid chromatin has a somewhat "loosened" structure perhaps related to the histone displacement that occurs in the transition of this cell stage to the next. In contrast, the relative stainability for tetraploid$_{1c}$ cells was generally lower than diploid$_{1c}$ cells (Figs. 2,3). These observations apparently are related to chromatin changes occurring during meiotic events. The Hl histone complements of germ cells in the rat testis are quite different from those of somatic tissues (28,4). Two Hl subtypes predominate in prepachytene spermatocytes and a third, Hlt, is introduced during pachytene and remains until the spermatid stage (4). Lennox and Cohn (23) have suggested that the unusual Hl subtype composition in prepachytene spermatocytes favors chromatin conformations compatible with the precise chromosomal pairing necessary for genetic recombination and also that it may expedite histone replacement during spermiogenesis.

These data show dramatic differences in the stainability of eight different dyes at various stages of sperm cell differentiation. By using one or several of these dyes, it should be feasible to monitor hypothetical abnormal protein transition when animals or humans (10) are exposed to agents that perturb spermatogenesis.

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LITERATURE CITED

1. Balhorn R: A model for the structure of chromatin in mammalian sperm. Cell Biol 93:298–305, 1982.
2. Balhorn R, Gledhill BL, Wyrobek AJ: Mouse sperm chromatin proteins: quantitative isolation and partial characterization. Biochemistry 16:4074–4080, 1977.
3. Balhorn R, Weston S, Thomas C, Wyrobek A: DNA packaging in mouse spermatids. Exp Cell Res 150:298–308, 1984.
4. Bucci LR, Brock WA, Meistrich ML: Distribution and synthesis of histone 1 subfractions during spermatogenesis in the rat. Exp Cell Res 140:111–118, 1982.
5. Calvin HI, Bedford SM: Formation of disulphide bonds in the nucleus and accessory structure of mammalian spermatozoa during maturation in the epididymis. J Reprod Fertil (Suppl) 13:65, 1971.
6. Clausen OPF, Parvinen M, Kirkhus B: Stage-related variations in DNA fluorescence distribution during rat spermatogenic cycle measured by flow cytometry. Cytometry 2:421–425, 1982.
7. Darzynkiewicz Z, Gledhill BL, Ringertz NR: Changes in deoxyribonucleoprotein during spermiogenesis in the bull. ^{3}H-actionomycin D binding. Exp Cell Res 58:435–438, 1969.
8. Darzynkiewicz Z, Traganos F, Kapuscinski J, Staiano-Coico L, Melamed MR: Accessibility of DNA *in situ* to various fluorochromes: Relationship to chromatin changes during erythroid differentiation of Friend leukemia cells. Cytometry 5:355–363, 1984.
9. Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR: Lymphocyte stimulation: A rapid multiparameter analysis. Proc Natl Acad Sci USA 73:2881-2884, 1976.
10. Evenson DP: Male germ cell analysis by flow cytometry: Effects of cancer, chemotherapy and other factors on testicular function and sperm chromatin structure. In: Proceedings of the conference on Clinical Cytometry, Andreeff MA (ed). NY Acad Sci (in press).
11. Evenson DP, Darzynkiewicz Z, Melamed MR: Comparison of human and mouse sperm chromatin structure by flow cytometry. Chromosoma 78:225–238, 1980.
12. Evenson DP, Darzynkiewicz Z, Melamed MR: Relation of mammalian sperm chromatin heterogeneity to fertility. Science 210:1131–1133, 1980.
13. Evenson DP, Higgins PH, Grueneberg D, Ballachey B: Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 6:238–253, 1985.
14. Evenson DP, Melamed MR: Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J Histochem Cytochem 31:248–253, 1983.
15. Gledhill BL, Campbell GL: Microfluorimetric comparison of chromatin during cytodifferentiation. In: Fluorescence Techniques in Cell Biology, Thaer AA, Sernetz M (eds). Springer-Verlag, New York, 1973, pp 151–162.
16. Gledhill BL, Gledhill MP, Rigler R Jr., Ringertz NR: Changes in deoxyribonucleoprotein during spermiogenesis in the bull. Exp Cell Res 41:652–665, 1966.
17. Grimes SR, Meistrich ML, Platz, RD, Hnilica LS: Nuclear protein transitions in rat testis spermatids. Exp Cell Res 110:31–39, 1977.
18. Hacker, U, Schumann J, Gohde W: Effects of acute gamma-irradiation on spermatogenesis as revealed by flow cytometry. Acta Radiol Oncol 19:361–368, 1980.
19. Hnilica LS: The Structure and Biological Function of Histones. CRC Press, Cleveland, 1972.
20. Hollstein U: Actinomycin. Chemistry and mechanism of action. Chem Rev 74:625–652, 1974.
21. Kierszenbaum AL, Tres LL: The packaging unit: A basic structural feature for the condensation of late cricket spermatid nuclei. J Cell Sci 33:265–283, 1978.
22. Latt SA: Fluorescent probes of DNA microstructure and synthesis. In: Flow Cytometry and Sorting, Melamed MR, Mullaney P, Mendelsohn M (eds). John Wiley and Sons, New York, 1979, pp 263–284.
23. Lennox RW, Cohen LH: The alterations in Hl histone complement during mouse spermatogenesis and their significance for Hl subtype function. Dev Biol 103:80–84, 1984.
24. Loir M, Lanneau M: An electrophoretic analysis of the basic nuclear proteins of ram spermatids. Exp Cell Res 92:509–512, 1975.
25. Mayer JR, Zirkin BR: Spermatogenesis in the mouse. I. Autoradiographic studies of nuclear incorporation and loss of ^{3}H amino acids. J Cell Biol 81:403, 1979.
26. McLean Grogan W, Farnham WF, Sabau JM: DNA analysis and sorting of viable mouse testis cells. J Histochem Cytochem 29:738–746, 1981.
27. Meistrich ML, Lake S, Steinmetz LL, Gledhill BL: Flow cytometry of DNA in mouse sperm and testis nuclei. Mut Res 49:383–396, 1978.
28. Seyedin SM, Cole RD, Krstler WS: Hl histones from mammalian testes. Exp Cell Res 136:399–405, 1981.
29. Zimmerman A, Truss F: The prognostic power of flow-through cytophotometric DNA determinations for testicular diseases. Anal Quant Cytol 2:247–251, 1980.
30. Zirkin BR, Soucek DA, Chong TS: Sperm nuclear packing and regulation during spermatogenesis and fertilization. Johns Hopkins Med J 151:101–112, 1982.

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